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Identification of Asteroid Genera With Species Capable of Larval Cloning

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Abstract. Asexual reproduction in larvae, larval cloning, is a recently recognized component of the complex life histories of asteroids. We compare DNA sequences of mitochondrial tRNA genes (Ala, Leu, Asn, Pro, and Gln) from larvae in the process of cloning collected in the field with sequences from adults of known species in order to identify asteroid taxa capable of cloning. Neighbor-joining analysis identified four distinct groups of larvae, each having no, or very little, sequence divergence (p distances ranging from 0.00000 to 0.02589); thus, we conclude that each larval group most likely represents a single species. These field-collected larvae cannot be identified to species with certainty, but the close assemblage of known taxa with the four larval groups indicates generic or familial identity. We can assign two of the larval groups discerned here to the genera *Luidia* and *Oreaster* and another two to the family Ophidiasteridae. This study is the first to identify field-collected cloning asteroid larvae, and provides evidence that larval cloning is phylogenetically widespread within the Asteroidea. Additionally, we note that cloning occurs regularly and in multiple ways within species that are capable of cloning, emphasizing the need for further investigation of the role of larval cloning in the ecology and evolution of asteroids.

Introduction

With the discovery of larval cloning (Bosch, 1988, 1992; Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994), new complexity has been recognized in the diverse developmental modes exhibited by asteroid echinoderms (sea stars). Nearly all asteroids reproduce sexually and have complex life cycles in which larval stages, having very different morphologies and habits from the adults, alternate with adult stages (Mortensen, 1921; Hyman, 1955; Chia and Walker, 1991). Asexual reproduction by adults is prevalent in some asteroid groups (*e.g.*, *Linckia*, *Coscinasterias*) and supplements the product of sexual reproduction by increasing the number of individuals derived from a given lineage. Asexual reproduction by larvae, larval cloning, is poorly understood, including which species are capable of it and what role it might play in the ecology and evolution of asteroids.

Three distinct modes of larval cloning have been observed in planktotrophic asteroid larvae collected from the field and reared in the laboratory (Bosch, 1988; Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994; Vickery and McClintock, 2000; Kitazawa and Komatsu, 2001). These modes—paratomy of the posterolateral arms, autotomization of the preoral lobe, and budding from the larval body and arm tips—share in common a period of dedifferentiation of larval tissues that are then redifferentiated in the clone (see Jaeckle, 1994, for details). Larval cloning in benthic, brooded, and pelagic lecithotrophic larvae has not been observed but may occur through some as yet unrecognized process. Clones are able to develop to and through metamorphosis and may themselves exhibit larval cloning (EJB and WBJ, pers. obs.; Vickery and McClintock, 2000; Kitazawa and Komatsu, 2001). However, it is not known whether juveniles derived from cloned larvae will develop to sexual maturity, or if larval cloning has fitness consequences for either the primary or cloned larvae. Larval

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cloning could possibly have a significant impact on asteroid life history by altering such parameters as dispersal, number of individuals, or fitness, thus emphasizing the need to identify those asteroid species capable of larval cloning.

Asteroid larvae in the process of cloning have been collected from portions of the tropical and subtropical western Atlantic Ocean (Jaeckle, 1994), including the Sargasso Sea (Bosch, 1988; Bosch *et al.*, 1989), and from the Bay of Bengal (Rao *et al.*, 1993). These larvae can be very common in collections, constituting from 10% to 90% of the asteroid larvae present (Bosch, 1988; Bosch *et al.*, 1989; Jaeckle, 1994; EJB and WBJ, pers. obs.). However, field-collected cloning larvae have not been identified specifically. Initially, cloning larvae were thought to be restricted to species in the genus *Luidia*, which have bipinnaria larvae that lack a brachiolar complex and are, in some *Luidia* species, quite large (Wilson, 1978; Domanski, 1984; Bosch *et al.*, 1989). But Bosch (1992) and Jaeckle (1994) showed that larval cloning is not taxonomically restricted when they reported larval cloning in brachiolaria larvae, which are common to all asteroid orders except the Paxillosida (to which *Luidia* belongs).

Cloning larvae have been observed in laboratory cultures as well. Previous laboratory studies have noted larval cloning in members of the Paxillosida and Forcipulatida (EJB and WBJ, pers. obs.; Vickery and McClintock, 2000; Kitazawa and Komatsu, 2001). These occurrences, in species found in the north Pacific, indicate that larval cloning may be more widely distributed geographically than previously recognized. However, field-collected cloning larvae have not been reported in these areas, and whether larval cloning occurs naturally in these species (*i.e.*, outside the laboratory) is not known.

Our goal is to identify asteroid larvae capable of cloning by comparing the DNA sequences of unknown, field-collected cloning larvae and known adult species. Because most asteroid larvae are morphologically similar, field-collected larvae can rarely be identified to family level, much less to genus or species, by visual inspection of morphological characteristics. Indirect identification of asteroid larvae based on correlations with geographical distributions of adults is unlikely because larvae may have great dispersal potential and do not necessarily remain close to their parental population (Thorson, 1961; Strathmann, 1974; Scheltema, 1986). Morphological identification of juveniles after metamorphosis is possible, but not always practical or dependable. Asteroid larvae are sensitive to laboratory culturing conditions (Strathmann, 1987), can take weeks to develop (*e.g.*, Komatsu *et al.*, 1991), and can delay metamorphosis for several months if a suitable settlement cue is not found (Pechenik, 1990). Often, laboratory cultures die before the larvae reach metamorphosis or before juveniles are large enough to be identified. As an alternative to culturing methods, we have used DNA sequence similarity to identify field-collected cloning asteroid larvae. DNA sequencing techniques are universally known and easily implemented in the laboratory for a quick assessment of potential larval identity. Identity then can be verified with more time-consuming laboratory culturing techniques.

Materials and Methods

We investigated the identity of field-collected cloning asteroid larvae by comparing the DNA sequences of five mitochondrial tRNA genes (Ala, Leu, Asn, Pro, and Gln) from the larvae to complementary sequences from adults of known species. The suitability of this gene region for species identification was initially assessed by comparing sequences obtained from a single known larva of *Luidia clathrata* to sequences from related, known adult asteroids. Comparison of sequences from the *L. clathrata* larva, a *L. clathrata* adult from a different locality, other *Luidia* species, and species from the closely related genera *Astropecten* and *Ctenodiscus* are shown in Table 1. The sequences obtained for the *L. clathrata* larva and adult are identical. However, significant nucleotide changes are observed between *L. clathrata* (larva and adult) and other *Luidia* species, as well as between *L. clathrata* (larva and adult) and species in other genera, which are reflected in genetic distances among species (Table 2).

Larvae used in our comparisons were collected from the tropical and subtropical western Atlantic Ocean, specifically from off the western shore of Barbados (31° 2″ N, 59° 4″ W) and from the Gulf Stream off the eastern shore of Florida (27.3° N, 79.6° W), by EJB and WBJ. Cloning larvae have been collected consistently and in large numbers at the Gulf Stream site (EJB and WBJ, pers. obs.). Individual larvae were scored for the presence and type of cloning exhibited. Most of the larvae were cloning by paratomy, but one was cloning by autotomy of the preoral lobe. Those that were not cloning (14 of 65) were similar to cloning larvae found in the same or in other collections, and so were assumed to have the ability to clone (Fig. 1). All larvae were preserved in 95% EtOH and shipped to SUNY Stony Brook for processing.

The five mitochondrial tRNA genes of interest (Ala, Leu, Asn, Pro, and Gln) were amplified from individual larvae using the polymerase chain reaction (PCR) and published echinoderm-specific primers (Smith *et al.*, 1993; Hart *et al.*, 1997). A typical total genomic DNA extraction was avoided because these extraction techniques often require a large amount of starting material. Instead, the entire larval body (first air-dried to remove traces of EtOH) was used in PCR as the DNA template (Medeiros-Bergen *et al.*, 1995). The thermocycling conditions of PCR are severe enough to disrupt the larval cells, releasing their DNA. Amplifications

Table 1

Alignment of tRNA sequences (Ala, Leu, Asn, Pro, and Gln) showing complete sequence identity between Luidia clathrata *adult and larva; sequence differences between* Luidia clathrata *and other* Luidia *species (*), other closely related genera (+), or both (^) are marked*

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                Ala
Luidia clathrata          -GTGAATTTAGTTTAAAA-GAA--AAAACCTTTGATTTGCATTCAAAAA----------A-TTTAGGT--TTAAGACCTAAAATTTACA---
L. clathrata larva        -GTGAATTTAGTTTAAAA-GAA--AAAACCTTTGATTTGCATTCAAAAA----------A-TTTAGGT--TTAAGACCTAAAATTTACA---
L. magellanica            -GTGAATTTAGTTTAACA-GAA--AAAACATTTGATTTGCACTCAAACA----------A-TTTAGGT--TTAAACCCTAAAGTTTACA---
L. foliolata              -GTGAATTTAATTTAAAA-GAA--AAAATATTTGATTTGCATTCAAACA----------A-TTTAGGT--TTAAAGCCTAAAGTTCACA---
L. alternata              -GTGAATTCAGTTTAAGA-GAA--AAAACTTTTGACTTGCATTCAAAAA----------A-TTTAGGT--TTAACTCCTAAAATTTACA---
Astropecten               -GTGAATTTAGTTTAAAA-GAC--AAAACATTTGATTTGCATTTAAAAA----------A-TCCAGGT--TTAATTCCTGGAATTCACA---
Ctenodiscus               -GTGAATTTAGTTTAAAA-GAT--AAAACATTTAATTTGCATTTAAAAA----------C-TTCAAGT--TTAACCCCTGAAATCCACA---
                                * *     *    +      *^   + *     * +  *                  +  ++          ^^    ++ * +^
                Leu
Luidia clathrata          -GCTAAAATAGCAAAGTG-GTA--AATGCTGTAGATTTAGGTTCTATTA----------T-CAAAGGTTCAAATCCTTTTTTTAGTT---
L. clathrata larva        -GCTAAAATAGCAAAGTG-GTA--AATGCTGTAGATTTAGGTTCTATTA----------T-CAAAGGTTCAAATCCTTTTTTTAGTT---
L. magellanica            -GCTAGAATAGCAAAGGG-GTA--AATGCAATAGATTTAGGATTTATTA----------T-CAAAGGTTCAATTCC-TTTTTTAGTT---
L. foliolata              -GCTAAAATAGCAAAGTG-GTA--AATGCAATAGATTTAGGATTTATTA----------C-CAAAGGTTCAATTCCTTTTTTTAGTT---
L. alternata              -ACTTAGGTAGCAAAGCG-GTA--AATGCGGTAGATTTAGGATCTATTA----------T-CAGGGGTTCGATTCTCTTCCTTAGTT---
Astropecten               -GTTAGAATAGCAAAGGG-GAA--AATGCAATAGATTTAGGATCTGTCA----------T-CAAGAGTTCGAGTCTCTTTTCTAGTT---
Ctenodiscus               -ACTGAGGTAGCAAAGTG-GTG--AATGCGGCAGATTTAGGATTTGTTA----------T-CAAGGGTTCTAATCCCTTTCTTAGTT---
                           ^+ ^^^^       ^   ++      ^^+        ^ ^ + +              *   *^+    ^ ^  ^^  *^+
                Asn
Luidia clathrata          -TGGGTTGTAGCCTAGT--GGA--AAGGCAACTGGCCGTTAACCAGGAG----------ATAACAAGATCAATACTTGTCAACTCAG---
L. clathrata larva        -TGGGTTGTAGCCTAGT--GGA--AAGGCAACTGGCCGTTAACCAGGAG----------ATAACAAGATCAATACTTGTCAACTCAG---
L. magellanica            -TGGGTTGTAGCCTAGC--GGA--AAGGCAACTGGCCGTTAACCAGGAG----------ATAACAAGATCAATACTTGTCAACTCAG---
L. foliolata              -TGGGTTGTAGCCTAGT--GGA--AAGGCAACTGGCCGTTAACCAGGAG----------ATAACAAGATCAATACTTGTCAACTCAG---
L. alternata              -TGGGTTGTAGCCTAGT--GGA--AAGGCAACTGGCCGTTAACCAGGAG----------ATAACAAGATCAATACTTGTCAACTCAG---
Astropecten               -TGGGTTGTAGCCTAAT--GGA--AAGGCAATTGGCCGTTAACCAGGAG----------ATAGTAAGATCAATACTTACCAACTCAG---
Ctenodiscus               -TGGGTTGTAGCCTAGT--GGA--AAGGCAATTGGCCGTTAACCAGGAG----------ATAACAAGATCAATACTTGTCAACTCAG---
                                    +*              +                                  ++             ++
                Pro
Luidia clathrata          -CAGAGAATAGTTTAATT-TAG--AGAATTGTAACTTTGGGAGTTATTG----------G-TACAAATATA-GAGTTTTGTTTCTCTGA---
L. clathrata larva        -CAGAGAATAGTTTAATT-TAG--AGAATTGTAACTTTGGGAGTTATTG----------G-TACAAATATA-GAGTTTTGTTTCTCTGA---
L. magellanica            -CA?AGAATAGTTTAATT-TAAA-AGAATTGTAACTTTGGGAGTTATTG----------G-TGCAAATGTA-AAGTTTTGTTTCTCTGA---
L. foliolata              -CAGAGAATAGTTTAACA-TAAA-AGAATTATAACTTTGGGAGTTATAG----------G-TGCAAATGTA-GAGTTTTGTCTCTCTGA---
L. alternata              -CAGAGAATAGTTTAGTT-T----AGAATAATAACTTTGGGAGTTATTG----------G-TGCAAATATA-GAGTTTTGTTTCTCTGA---
Astropecten               -CAGAAAATAGTTTAATT------AGAATAATAACTTTGGGAGTTATTG----------G-TGTAAATATA-GAGTTTTATTTTTCTGA---
Ctenodiscus               -CAGGAAATAGTTTAATA------AGAATGATAGCTTTGGGAGTTGTTA----------G-TGTAAATATG-GAATTTTACTTTTCTGA---
                              ++          *^  +^^*       ^^   +                        ^+    * + * +    ++* +
                Gln
Luidia clathrata          -TAGAAAGTAGTATAAT--GGA--ATTACAAAGATCTTTGACTTCTTAA----------A-TATAAGTTCAATCCTTATCTTTCTAA---
L. clathrata larva        -TAGAAAGTAGTATAAT--GGA--ATTACAAAGATCTTTGACTTCTTAA----------A-TATAAGTTCAATCCTTATCTTTCTAA---
L. magellanica            -TAGAAAGTAGTATAAA--GGT--ATTACAAAGATCTTTGACTTCTTAA----------A-CATAAGTTCAATTCTTATCTTTCTAA---
L. foliolata              -TAGAAAGTAGTATAAA--GGC--ATTACAAAGATCTTTGACTTCTTAA----------A-CATAAGTTCAATTCTTATCTTTCTAA---
L. alternata              -TAGAAAGTAGTATAGGC-GGA--ATTACAAAGACCTTTGACCTCTTAA----------A-CATAAGTTCAACTCTTGTCTTTCTAA---
Astropecten               -TAGAAAGTAGTATAAT--GGT--AAAACAAAGAACTTTGACTTCTTTA----------A-TATAAGTTCAATTCTTATCTTTCTAA---
Ctenodiscus               -TAGAAAGTAGTATAAC--GGC--AATACATAGAACTTTGATTTCTTAA----------C-TACAAGTTCAATTCTTGTCTTTCTAA---
                               *^*   ^   ++   +   ^      +*    +                    + * +       *^   ^
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Table 2

Genetic distances calculated using methods for uncorrected (p) *distance between tRNA genes from a known* Luidia clathrata *larva, other* Luidia *species and species from closely related genera*

	1	2	3	4	5	6	7
1. *Luidia clathrata*	----						
2. *Luidia clathrata* larva	0.00000	----					
3. *Luidia magellanica*	0.06422	0.06422	----				
4. *Luidia foliolata*	0.06948	0.06948	0.04450	----			
5. *Luidia alternata*	0.09243	0.09243	0.10482	0.11230	----		
6. *Astropecten*	0.12597	0.12597	0.12657	0.13699	0.14601	----	
7. *Ctenodiscus*	0.14561	0.14561	0.16100	0.15135	0.14592	0.14566	----

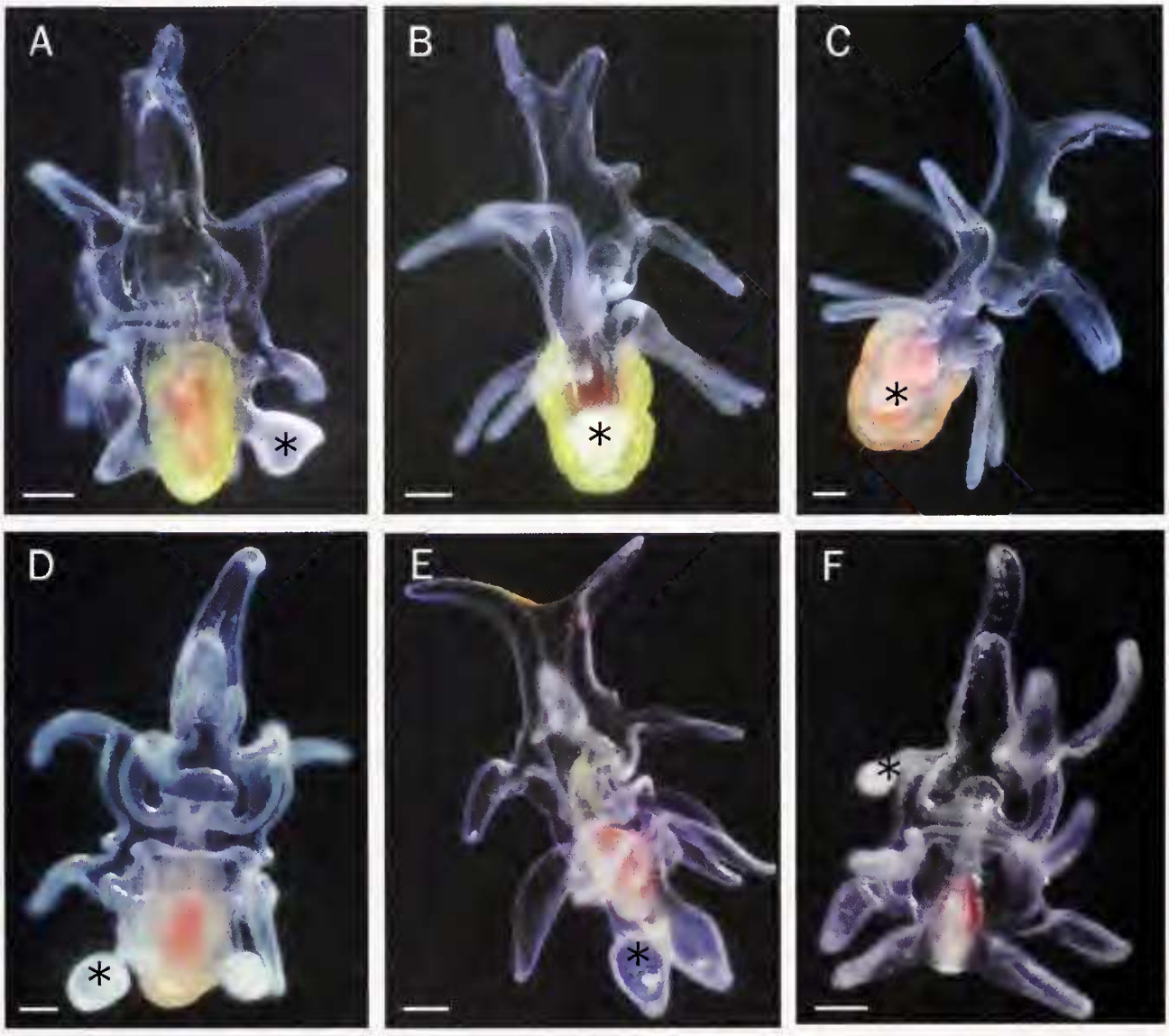

Figure 1. Representative asteroid larvae with clones collected from plankton samples taken off the eastern coast of Florida. Obvious morphological characters such as color and arm length appear to be labile and are unreliable as taxonomic characters. Definitive morphotypic characters distinguishing species have not yet been identified. (A-D) Brachiolariae included in larval group 1. (E) Bipinnaria of larval group 2, which includes at least one *Luidia* species. (F) Bipinnaria from larval group 3/4. Asterisks indicate larval clones. Scale bars = 150 μm.

were successful for 44 out of 65 (68%) larvae collected. Amplification reactions were carried out in 25-μl volumes of a standard reaction mix with Taq DNA polymerase (GIBCO Life Technologies) using a MJ Research PTC-200 thermocycler. All samples were purified in 2% NuSieve agarose (FMC BioProducts) and gel extracted (QIAGEN or GIBCO Life Technologies kits). Purified samples were chemically transformed (Brown, 1991) into XLI Blue (Stratagene) competent cells using pGEM-T vector (Promega). Cloned samples were purified with Wizard *Plus* Miniprep purification kit (Promega), sequenced in forward and reverse directions using vector sequence primers [M13 (−20) M13 (rev)] and dye-terminator sequencing reaction mix, and analyzed with an ABI 373 automated sequencer (PE Applied Biosystems). Sequence data from both strands were combined and edited with Sequencher 3.0 (Gene Codes Corporation, 1995) and aligned by eye in accordance with established tRNA alignment of other taxa based on the molecular structure of tRNAs (Sprinzl *et al.*, 1998). Larval sequences were then aligned to similarly aligned sequences

of the same genes from known adult asteroid species (Himeno *et al.*, 1987; Hart *et al.*, 1997; Knott and Wray, 2000; KEK, unpubl. data). Regions between the tRNA genes were also sequenced. These sequences were variable among the asteroid taxa and could not be aligned. Since an assessment of homology of the inter-gene sequences could not be made, they were eliminated from the data set.

The combined aligned data set was analyzed with multiple distance criteria using PAUP* ver. 4.0b4a (Swofford, 1998) to assess the degree of sequence similarity. Sequence differences were calculated first as uncorrected (*p*) distance, a measure of the number of aligned sequence positions containing non-identical nucleotides divided by the total number of positions compared. Genetic distance was then calculated using modified methods that account for superimposed mutational events according to the Jukes-Cantor and Kimura 2-parameter models of evolutionary change (described in Swofford *et al.*, 1996). Genetic distances obtained from all methods were then used in neighbor-joining analysis. Differences in trees obtained using different genetic distance calculations were assessed with the Kishino and Hasegawa (1989) and Templeton (1983) nonparametric tests in PAUP*. Stability of clades in the resulting trees was assessed by bootstrap analyses (1000 replicates; Felsenstein, 1985).

Results and Discussion

Neighbor-joining analysis using three methods of calculating genetic distances resulted in only slightly different tree topologies. Analysis of uncorrected (*p*) distances and Jukes-Cantor distances yielded identical tree topologies. Analysis of Kimura 2-parameter distances yielded a longer tree topology that was statistically different when tested with the Kishino and Hasegawa test ($P = 0.013$) and Templeton nonparametric test ($P = 0.013$). However, because none of the relationships in clades including cloning larvae were affected by the genetic distance calculation used, only the tree generated using uncorrected (*p*) distances is shown and discussed (Fig. 2).

Analysis of sequence similarity identified four distinct groups of cloning larvae. Within these groups there was very little sequence divergence (*p* distances ranging from 0.00000 to 0.02589), indicating that each group most likely represents a single species (Table 3). However, neighbor-joining analysis of the tRNA sequences did not place any of the 44 known adult asteroid species analyzed here within the larval groups (Fig. 2). The field-collected larvae thus cannot be identified to species with the limited number of sequences from known asteroid species available for comparison. However, the close assemblage of adults of known taxa with the four larval groups identifies those genera or families with species capable of larval cloning and indicates species likely to be capable of larval cloning. Representative larvae from the identified larval groups are shown in Figure 1. This is the first study to identify field-collected cloning larvae.

The largest group of cloning larvae (group 1; 24 individuals) has sequences with high similarity to those from two *Oreaster* species, which group basally to the larvae. Both larval group 1 and the *Oreaster* clade are well supported (bootstrap percentages: 100 and 73 respectively), as is the grouping of larval group 1 with the *Oreaster* clade (100% bootstrap support). *Oreaster reticulatus* is common in the tropical western Atlantic where the cloning larvae were collected, whereas *O. occidentalis* is found in the eastern Pacific. The only other species of *Oreaster* in the Atlantic Ocean is *O. clavatus*, found in the eastern Atlantic from Cape Verde to the Gulf of Guinea (Clark and Downey, 1992). *O. clavatus* was not included in this analysis, and may be a good candidate species for the identity of larval group 1. The genetic distance observed between the *Oreaster* species and larval group 1 is not large (range: 0.06745 to 0.10026; average: 0.08262) and is comparable to other intra-genus distances (Table 3). However, some very closely related genera (within the same family) have genetic distances as low as that seen between larval group 1 and the *Oreaster* clade. Thus, it is also possible that the larvae could belong to a species in a genus closely related to *Oreaster*. If so, the species identity of larval group 1 must lie with a very close relative of *Oreaster*, perhaps within its taxonomic family, the Oreasteridae, or within another closely related family, the Asteropseidae (Blake, 1987).

Generic identification is more certain for a smaller group of cloning larvae (group 2; 5 individuals) and a single cloning larva, both of which fall within a clade of *Luidia* species (96% bootstrap support). There are seven *Luidia* species in the tropical and subtropical western Atlantic Ocean (Clark and Downey, 1992). Our analysis includes two of these, as well as other *Luidia* species from the Pacific Ocean. The western Atlantic species not represented here are good candidates for the species identity of larval group 2 and the single cloning larva contained in this clade. Relationship of the five individuals in larval group 2 is well supported, in 100% of bootstrap replicates. The single cloning larva does not group with larval group 2, and instead is unresolved in a polytomy with the Pacific *Luidia* species. The grouping of this individual with the Pacific species is only moderately supported with bootstrap analyses (68%), so its affinities to other *Luidia* species are unclear. However, genetic distance between this individual and larval group 2 is high (averaging 0.08958). Since genetic distances within larval group 2 are over 30 times lower (average: 0.00278), it is unlikely that the single cloning larva is also member of this group. Likewise, genetic distances between known *Luidia* species and larval group 2 are also high (range: 0.07524

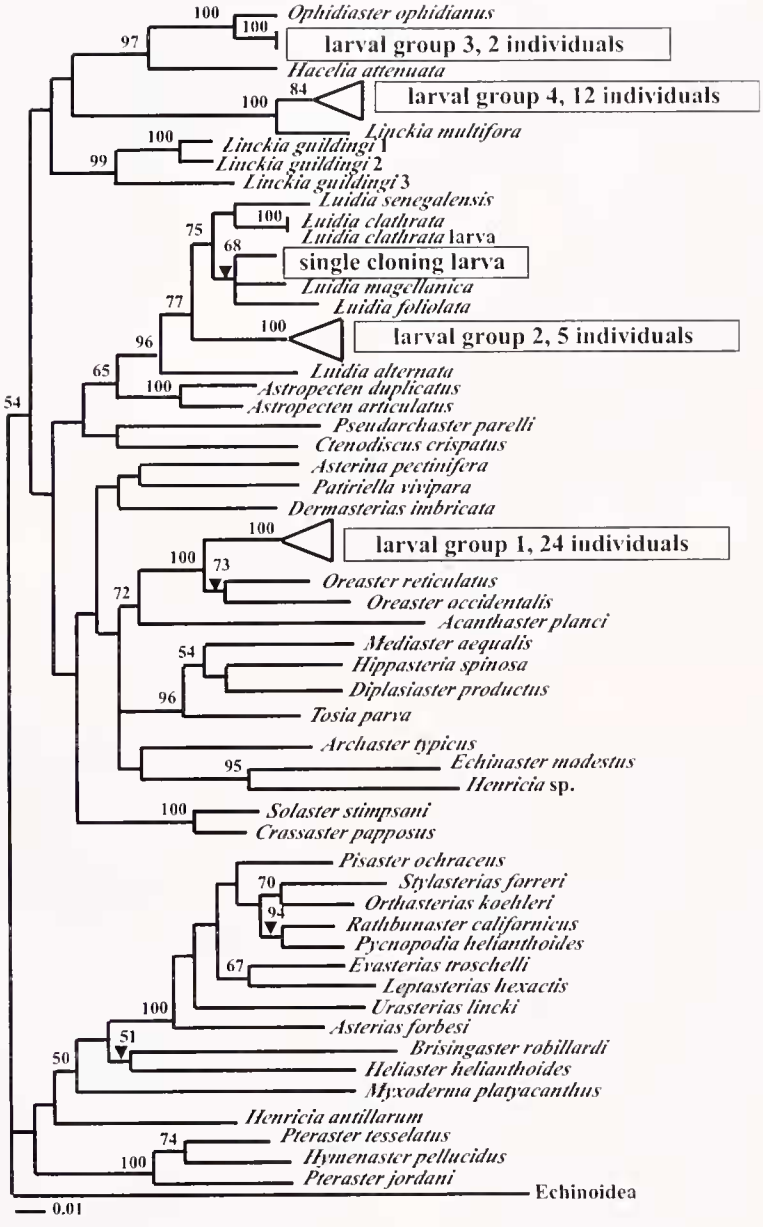

Figure 2. Phylogenetic tree resulting from neighbor-joining analysis of uncorrected (*p*) distances between mitochondrial tRNA sequences of known asteroid species and field-collected cloning larvae. Field-collected cloning larvae fall into four distinct groups and one single cloning larva (boxed), which are phylogenetically widespread. Numbers of larvae in each larval group are indicated. Numbers at nodes within the tree are bootstrap percentages from 1000 replicates. Larval tRNA sequences are accessioned in GenBank under numbers AY249946–AY249978. GenBank accession numbers for known asteroid taxa include some published previously (Himeno *et al.*, 1987; Hart *et al.*, 1997; Knott and Wray, 2000) and AY245490–AY245506.

to 0.11438), similar to those seen between species of the same genus (Table 3). The sequence differences between larval group 2, the single cloning larva, and other *Luidia* species suggests that multiple species within *Luidia* are capable of larval cloning. The alternative, that there may be genetic variation within species complicating our similarity analyses, is not likely given that intra-species genetic distances determined in this and other studies are very low (Table 3). Sequences from a known larva of *L. clathrata* and an adult representative of this species from a different locality are identical and group together in neighbor-joining analysis with 100% bootstrap support (Tables 2 and 3).

The remaining two groups of cloning larvae (group 3 with 2 individuals and group 4 with 12 individuals) show

Table 3

Genetic distances observed between asteroid taxa at different taxonomic levels; genetic distance reported is calculated from tRNA genes (*) or the COI gene (°)

Taxonomic Level	Species	Distance	Reference
Intra-species	Larval group 1	0.00000*	This paper
	Larval group 2	0.00278*	This paper
	Larval group 3	0.00557*	This paper
	Larval group 4	0.00000*	This paper
	Luidia clathrata	0.00000*	This paper
	Linckia guildingi	0.00000°	Williams 2000
	Patiriella brevispina	0.00843*	Hart *et al.* 1997
Intra-genus	Larval group 1 and *Orcaster* species	0.08262*	This paper
	Larval group 2 and single cloning larva	0.08958*	This paper
	Larval group 3 and *Ophidiaster* species	0.03354*	This paper
	Luidia clathrata and *Luidia altemata*	0.09243*	This paper
	Linckia guildingi and *Linckia laevigata*	0.15940°	Williams 2000
	Oreaster reticulatus and *Oreaster occidentalis*	0.07812*	Hart *et al.* 1997
	Asterina gibbosa and *Asterina miniata*	0.10050*	Hart *et al.* 1997
Inter-genus	*Luidia clathrata* and *Astropecten articulatus*	0.11293*	This paper
	Linckia guildingi and *Fromia indica*	0.21626°	Williams 2000
	Asterina gibbosa and *Patiriella brevispina*	0.10687*	Hart *et al.* 1997
	Oreaster reticulatus and *Asterinia miniata*	0.14916*	Hart *et al.* 1997

affinities to the asteroid family Ophidiasteridae. The two cloning larvae in larval group 3 have very low genetic distance (0.00557), and their relationship is well supported with bootstrap analyses (100% of replicates). These larvae are most closely related to *Ophidiaster ophidianus*, a species that ranges in the eastern Atlantic from the Azores to the Gulf of Guinea (Clark and Downey, 1992). Most likely, *O. ophidianus* larvae would not be collected in the western Atlantic Ocean, so the larvae in group 3 probably belong to some other *Ophidiaster* species. Genetic distances between *O. ophidianus* and the cloning larvae of group 3 (0.02796 and 0.03354) are higher than intra-species genetic distances for other asteroids (Table 3), giving more evidence that their species identity is probably not *O. ophidianus*, but some other close relative. The other "ophidiasterid" group of cloning larvae (group 4; 12 individuals) is strongly supported with bootstrap analyses (84% of replicates). Together, the larvae group with the species *Linckia multifora* (100% bootstrap support). *L. multifora* does not exist in the Atlantic Ocean (Clark and Downey, 1992), so the larvae examined here are not expected to be members of this species. *Linckia guildingi* specimens collected from three localities also included in this study do not group with *L. multifora* and larval group 4 directly, as would be expected for species in the same genus. Instead, these species group basally to all the ophidiasterid taxa, a grouping that is not supported by bootstrap analyses. Genetic distances among the 12 individuals in larval group 4, and between these larvae and other ophidiasterid species, are shown in Table 4. Certainly, additional species should be sequenced to test these relationships, particularly other *Linckia* species not sampled in this study.

One of the larval groups identified here (group 1; 24 individuals), has members that display different modes of larval cloning (paratomy and autotomy). Although different modes of cloning were not exhibited by individuals simultaneously, the species represented by larval group 1, identified here, has the capability to reproduce asexually in multiple ways. The fact that a single species is capable of multiple modes of larval cloning has been reported for laboratory-cultured species (Vickery and McClintock, 2000; Kitazawa and Komatsu, 2001) but not for field-collected individuals.

Our results also indicate that larval cloning occurs regularly within asteroid species. The three larval groups containing more than two individuals (groups 1, 2, and 4) are composed of larvae that were collected at different localities (Barbados and Florida). In addition, cloning larvae with morphological types identical to those studied here have been collected at these sites over multiple years. Regular occurrence of larval cloning is implied in a recent experimental study of larval cloning in the laboratory. Vickery and McClintock (2000) show that cloning occurs in laboratory-cultured *Pisaster ochraceus* only at temperatures and food regimes similar to those the larvae would encounter in the field, rather than of extremes of temperature and food abundance or composition. We expect that the regular occurrence of larval cloning is more common than sporadic cloning in response to environmental variation. The effects

Table 4

Genetic distances between the 12 individuals in larval group 4 and closely related taxa in the Ophidiasteridae, calculated using methods for uncorrected (p) *distance*

TAXA	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.
1. larva 1	----															
2. larva 2	0.0028	----														
3. larva 3	0.0028	0.0057	----													
4. larva 4	0.0000	0.0029	0.0028	----												
5. larva 5	0.0000	0.0029	0.0028	0.0000	----											
6. larva 6	0.0028	0.0057	0.0057	0.0028	0.0028	----										
7. larva 7	0.0028	0.0057	0.0057	0.0028	0.0028	0.0057	----									
8. larva 8	0.0000	0.0028	0.0028	0.0000	0.0000	0.0028	0.0028	----								
9. larva 9	0.0057	0.0085	0.0085	0.0057	0.0057	0.0085	0.0085	0.0057	----							
10. larva 10	0.0028	0.0029	0.0058	0.0028	0.0028	0.0057	0.0057	0.0028	0.0087	----						
11. larva 11	0.0057	0.0085	0.0085	0.0057	0.0057	0.0085	0.0085	0.0057	0.0113	0.0086	----					
12. larva 12	0.0172	0.0201	0.0201	0.0172	0.0172	0.0201	0.0201	0.0172	0.0230	0.0203	0.0230	----				
13. *Linckia multifora*	0.0398	0.0427	0.0427	0.0398	0.0398	0.0426	0.0426	0.0398	0.0456	0.0432	0.0455	0.0549	----			
14. *L. guildingi* 1	0.2170	0.2199	0.2198	0.2170	0.2170	0.2169	0.2199	0.2170	0.2227	0.2228	0.2228	0.2170	0.2287	----		
15. *Hacelia attenuata*	0.2027	0.2056	0.2054	0.2027	0.2027	0.2026	0.2055	0.2027	0.2084	0.2053	0.2083	0.2083	0.2226	0.1552	----	
16. *Ophidiaster ophidianus*	0.1998	0.2026	0.2026	0.1998	0.1998	0.1997	0.2027	0.1998	0.2054	0.2024	0.2056	0.2026	0.2195	0.1581	0.1032	----

of regular cloning on asteroid life history and population dynamics are unknown.

Conclusions

Identification of asteroid species capable of larval cloning is an important first step for continued study of this unusual reproductive strategy, and we have shown that field-collected cloning larvae can be identified using molecular techniques. Beyond a broad understanding of the morphological changes involved in larval cloning, very little is known about the processes of cloning or its role, if any, in the ecology and evolution of asteroids (Jaeckle, 1994). Once field-collected cloning larvae are identified, experiments for determining the role of larval cloning in asteroid life history, population dynamics, and developmental evolution can be pursued.

Our results indicate that there are four (possibly five) species capable of larval cloning in the tropical/subtropical western Atlantic Ocean. Species cannot be definitively identified at this time, but we can tentatively assign two of the larval groups discerned here to the genera *Luidia* and *Oreaster* and another two to the family Ophidiasteridae. Our identification of a *Luidia* species that is capable of larval cloning is not surprising. The initial description of paratomy in a field-collected larva was diagnosed to the asteroid genus *Luidia* on the basis of unique larval anatomical features (see Introduction). Our results, with a tentative identification to *Oreaster* and with larval groups falling outside the Paxillosida, support Bosch's (1992) and Jaeckle's (1994) observations that larval cloning is not restricted to *Luidia* and the Paxillosida. Within the asteroid family Ophidiasteridae, many species are capable of asexual reproduction as adults, particularly *Linckia*. The presence of larval cloning in species that also alternate between sexual and asexual reproduction as adults would be a complex twist to more typical asteroid life-history strategies.

Although Lacalli (2000) has claimed that larval cloning in asteroids is not common, our results indicate the opposite. This phenomenon has most likely been overlooked by echinoderm biologists, and as yet we cannot be sure how common it is. For example, clones in laboratory cultures may appear to be malformed embryos resulting from irregular development or unusual laboratory conditions and thus disregarded. Similarly, field-collected cloning larvae or developing clones may be misinterpreted as individuals that were damaged during collection (Bosch *et al.*, 1989). We feel certain that increased awareness by echinoderm biologists will produce more reports of larval cloning in asteroids, and perhaps in other echinoderms. Cloning has already been observed in the Ophiuroidea, sister group to the Asteroidea (Balser, 1998). Species identifications are necessary for studying larval cloning in a phylogenetic context. Results presented here are the beginning of an ongoing evolutionary analysis of the possibly ancient origin of larval cloning, with losses in some asteroid groups; or alternatively, multiple origins of larval cloning within the Asteroidea. Certainly, evidence of parallel evolution of derived larval forms is common among marine invertebrates (Strathmann, 1978;

Wray, 1996; Hart, 2000). Despite being only recently confirmed in asteroids (Bosch *et al.*, 1989; Jaeckle 1994), larval cloning may have an ancient evolutionary origin.

The larval groups, or species, discerned here consist of multiple cloning larvae collected from different localities. Widespread occurrence of cloning larvae may not be surprising, since planktonic larvae of asteroids can disperse great distances. In addition, the adults of many asteroid species have broad geographic ranges that in some cases extend beyond the area sampled here. The identification of different modes of larval cloning within one species (larval group 1) is a bit more surprising, despite observations of multiple modes of cloning in laboratory-cultured species (Vickery and McClintock, 2000; Kitazawa and Komatsu, 2001). The modes of larval cloning observed here, paratomy and autotomy, are morphologically very distinct. They affect different regions of the larval body, and they lead to different developmental regimes for the resulting clones (Jaeckle, 1994). Our results imply that the different modes of larval cloning may be less distinct than previously thought and call for further investigation of the developmental mechanisms involved in larval cloning.

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